



## **Circulating blood cells and extracellular vesicles in acute cardioprotection**

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# **Title: Circulating blood cells and extracellular vesicles in acute cardioprotection**

**Short title:** Circulating cells and vesicles in cardioprotection

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## Abstract

During an ST-elevation myocardial infarction (STEMI), the myocardium undergoes a prolonged period of ischaemia. Reperfusion therapy is essential to minimize cardiac injury but can paradoxically cause further damage. Experimental procedures to limit ischaemia and reperfusion (IR) injury have tended to focus on the cardiomyocytes since they are crucial for cardiac function. However, there is increasing evidence that non-cardiomyocyte resident cells in the heart (as discussed in a separate review in this Spotlight series) as well as circulating cells and factors play important roles in this pathology. For example, erythrocytes, in addition to their main oxygen-ferrying role, can protect the heart from IR injury via the export of nitric oxide bioactivity. Platelets are well-known to be involved in haemostasis and thrombosis, but beyond these roles, they secrete numerous factors including sphingosine-1 phosphate (S1P), platelet activating factor (PAF) and cytokines that can all strongly influence the development of IR injury. This is particularly relevant given that most STEMI patients receive at least one type of platelet inhibitor. Moreover, there are large numbers of circulating vesicles in the blood, including microvesicles and exosomes, which can exert both beneficial and detrimental effects on IR injury. Some of these effects are mediated by the transfer of miRNA to the heart. Synthetic miRNA molecules may offer an alternative approach to limiting the response to IR injury. We discuss these and other circulating factors, focussing on potential therapeutic targets relevant to IR injury. Given the prevalence of co-morbidities such as diabetes in the target patient population, their influence will also be discussed. This article is part of a Cardiovascular Research Spotlight Issue entitled 'Cardioprotection Beyond the Cardiomyocyte', and emerged as part of the discussions of the European Union (EU)-CARDIOPROTECTION Cooperation in Science and Technology (COST) Action, CA16225.

## 1. [Introduction](#)

During an ST-elevation myocardial infarction (STEMI) the myocardium undergoes a prolonged period of ischaemia. Reperfusion therapy is essential to minimize cardiac injury but can paradoxically cause further damage.<sup>1</sup> Experimental procedures to limit ischaemia and reperfusion (IR) injury have been developed.<sup>1-3</sup> These strategies include ischaemic conditioning applied before ischaemia (preconditioning or IPC), after ischaemia (postconditioning or IPost) or to a distal organ or limb (remote conditioning, RIC). In addition, numerous pharmacological strategies activate either the PI3K/AKT (Reperfusion Injury Salvage Kinase, RISK), JAK/STAT (survivor activating factor enhancement or SAFE), or cGMP/PKG signalling pathways. These pathways have various effects on cardiomyocytes, but inhibition of the mitochondrial permeability transition pore (MPTP) has been described as a common end effector.<sup>1</sup> Furthermore, the mechanism of cardioprotection may also involve global changes in cardiac gene expression.<sup>4,5</sup>

Unfortunately, despite success in limiting IR injury in experimental animal models, the above approaches have not translated well to the clinical setting.<sup>1,6</sup> Possible reasons for this have been extensively discussed.<sup>1,2,6,7</sup> One reason is likely to be the prevalence of co-morbidities such as dyslipidaemia, diabetes and age in the STEMI patient population, which can impede cardioprotective strategies.<sup>8</sup> Another reason is that many patients are already taking drugs (e.g.: statins) or are administered drugs (e.g.: platelet P2Y<sub>12</sub> inhibitors) that are known to influence cardioprotection.<sup>8</sup> It may also be relevant that, because of their crucial role in cardiac function, most cardioprotection studies have focussed on protecting the cardiomyocytes. However, increasing evidence suggests that solely targeting cardiomyocytes may be insufficient to protect the heart in the complex scenario of STEMI, and a multi-target approach may be necessary.<sup>9</sup> In this regard, it may also be important to consider the roles played by innate immunity and inflammation, and the nervous system in addition to non-cardiomyocyte cells resident in the heart - topics which are discussed in a separate review in this Spotlight series.**(references to be added in proof)** Here, we address the importance of circulating blood cells and factors in IR injury and cardioprotection. We examine the role played by platelets, erythrocytes, as well as the extracellular vesicles (EVs) they release into the blood. While thrombosis is clearly a fundamental cause of coronary occlusion and myocardial ischaemia, factors targeting the thrombus and clotting factors may exert cardioprotective effects independent of occlusion. Furthermore, non-vesicular RNA may be an important cardioprotective approach. Lymphocytes play a complex role in IR injury. Circulating B- and T-lymphocytes are recruited to the injured myocardium in the days following infarction, and contribute to healing after AMI, but there is also some evidence that T cells contribute to acute myocardial IR injury. The role of lymphocytes and other immune cells is discussed in detail in an accompanying review in this series.**(reference to be added in proof)**

While there are certainly roles for circulating cells and factors in the longer time-scale of response to IR including inflammation and ventricular remodelling, we focus on their role in initial myocardial injury following acute IR injury.

## 2. [Platelets](#)

Platelets are small, anucleate cell fragments whose primary function is to initiate haemostasis in response to small vessel injury. However, they are emerging as important factors in the regulation of vascular homeostasis in many organs, including the heart. When activated, platelets can initiate haemostasis to prevent bleeding and eventually propagate a thrombus. STEMI is the consequence of coronary occlusion by thrombosis following plaque rupture. The latter promotes platelet adhesion and aggregation and thereby contributes to the blood clotting process. Platelets release various factors that may influence the heart during IR including cytokines, microRNAs (miRNAs), chemerin,

sphingosine-1-phosphate (S1P), and platelet-activating factor (**Figure 1**), thereby affecting the non-thrombogenic properties of the vascular endothelium and disturbing cardiomyocyte functions.

Activated platelets release a variety of factors that can affect IR injury. Some of these are vaso-active, as discussed further in an accompanying review.**(reference to be added in proof)** Other factors may act directly on cardiomyocytes and worsen their response to IR. For example, activated platelets release chemerin, an adipokine involved in inflammation, obesity, insulin resistance and metabolic syndrome.<sup>10</sup> Acting through chemokine-like receptor1 (CMKLR1 or ChemR23), it reduces AKT phosphorylation, activates caspase-9, and induces apoptosis in murine cardiomyocytes, which suggests it could also increase cardiac IR injury.<sup>11</sup>

Platelets also release numerous factors which may activate cardioprotective RISK and SAFE pathways. For example, they are a major source of CXCL12 (stromal cell derived factor -1 $\alpha$ , SDF-1 $\alpha$ ), which is released upon activation and can reduce IR injury in rodent and human myocardium, in addition to promoting longer time-scale repair mechanisms.<sup>12, 13</sup>

Platelet-derived S1P appears to make an important contribution to protection from IR injury. Platelets contain sphingosine kinase, which can transform membrane sphingosine into S1P for storage and release.<sup>14</sup> S1P can have both pro- and anti-aggregatory effects via G-protein coupled receptors (GPCRs) on platelets.<sup>14</sup> Importantly, S1P can also directly induce myocardial protection, apparently via S1P<sub>1</sub>, S1P<sub>2</sub> and S1P<sub>3</sub> receptors in cardiomyocytes, leading to activation of the RISK and SAFE pathways.<sup>15-18</sup> It has also been reported that PAK1/AKT/NOS3 signalling may mediate cardioprotection by S1P.<sup>18, 19</sup> Mice lacking both S1P<sub>2</sub> and S1P<sub>3</sub> receptors have 50% smaller infarcts after IR,<sup>18</sup> but it is not clear which cell type mediates this effect. Nevertheless, studies demonstrate that S1P is a pivotal mediator of cardioprotection and can trigger IPC and IPost. Indeed, S1P mediates powerful cardioprotection in isolated mouse hearts.<sup>20, 21</sup>

Diabetes can increase platelet hyperactivity and oxidative stress leading to cardiovascular complications.<sup>22</sup> These alterations may, at least in part, be responsible for the reduced ability to induce cardioprotection in models of uncontrolled diabetes.<sup>8</sup> Pre-treatment of isolated rat hearts with platelets from healthy subjects was protective against IR injury, whereas platelets from diabetic subjects were not, possibly due to altered release of S1P.<sup>23</sup>

Cardioprotective strategies such as IPC and IPost can induce the release of S1P.<sup>21, 24</sup> Whether these manoeuvres affect S1P release from platelets is not clear. Nevertheless, P2Y<sub>12</sub> inhibitors induce a conditioning-like cardioprotection that requires both platelets and S1P.<sup>25-27</sup> Platelets collected from patients with ACS increased injury when perfused through isolated rat hearts, and this cytotoxicity was blocked by P2Y<sub>12</sub> inhibitors.<sup>28</sup> However, prevention of platelet aggregation alone is not protective *in vivo*, since infarct size is unaltered in thrombocytopenic rats that remain untreated.<sup>27</sup> All P2Y<sub>12</sub> antagonists tested to date have been found to be cardioprotective in animals, and neither IPC nor IPost can add protection to that induced by the anti-platelet drugs.<sup>25, 29</sup> Since virtually all PCI-patients are treated with P2Y<sub>12</sub> inhibitors, it is important that future cardioprotective interventions are tested in an animal model receiving a P2Y<sub>12</sub> antagonist. The failure to clinically translate IPost-mimetics, which had appeared so protective in animal studies<sup>25, 29</sup> has led to the mistaken assumption that animal hearts are not appropriate models of human hearts. But even in animal studies, IPost was unable to confer further infarct size reduction in an animal concomitantly treated with a P2Y<sub>12</sub> antagonist.<sup>25</sup> It would therefore appear that in the presence of a P2Y<sub>12</sub> antagonist, further cardioprotection can be achieved only if the intervention has a different mechanism of protection from the platelet inhibitor.<sup>15, 25, 29, 30</sup> This approach should pave the way to translation of cardioprotective protocols into successful clinical treatments.

The phosphoglyceride Platelet Activating Factor (PAF) is produced and released by platelets, endothelial cells and leukocytes.<sup>31</sup> PAF acts as an autocrine/paracrine mediator on various cell types including cardiomyocytes, endothelial cells, smooth muscle cells, and platelets.<sup>31</sup> PAF has a dual role in IR.<sup>31</sup> IR causes the release of high quantities of PAF (1-10 nmol/L) with direct and indirect negative effects on coronary and cardiac functions, including a strong arrhythmogenic effect.<sup>31</sup> At very low concentrations (pM), PAF has a cardioprotective effect similar that that elicited by IPC.<sup>31-33</sup> Cardioprotection by PAF involves activation of the RISK kinase pathway, including PKC, AKT, and NOS.<sup>32</sup> Interestingly, a PAF-receptor antagonist impairs the infarct-sparing effect of both IPC and PAF.<sup>32</sup>

Although PAF or other endogenous factors within platelets may participate in triggering IPC-induced cardioprotection, they appear not to be required for cardioprotection by IPC, since thrombocytopenia did not abolish cardioprotection by IPC.<sup>15</sup> However, platelets might still affect infarction in patients with coronary artery disease or co-morbidities such as diabetes where platelets may be activated. Thus, platelets not only affect haemostasis and thrombosis, but platelet-derived products including EVs can have a profound effect on infarct size and cardioprotection.

### 3. Erythrocytes

It is well-known that erythrocytes are involved in the regulation of the cardiovascular system via mechanisms that include their interaction with the endothelium.<sup>34-36</sup> These mechanisms include the export of NO-like bioactivity and ATP that exert important cardiovascular effects. Additionally, erythropoietin (EPO), a kidney-derived cytokine that has the ability to increase red blood cell mass, can protect cardiomyocytes from apoptotic cell death through NOS3-derived NO production.<sup>37</sup> In the setting of IR, erythrocytes were originally suggested to protect the isolated rat heart from IR injury via a NOS-dependent mechanism.<sup>38</sup> This was supported by the observation that mice with blood cells lacking NOS3 had lower circulating nitrite and developed larger infarcts following IR than control mice, supporting a role of erythrocyte NOS3 under *in vivo* conditions.<sup>37, 39</sup> It was subsequently shown that export of NOS3-derived NO bioactivity from erythrocytes induced cardioprotection in the isolated heart.<sup>40</sup> This effect was tightly controlled by the enzyme arginase which is known to reciprocally regulate NO formation by competing with NOS3 for the substrate L-arginine. Consequently, inhibition of erythrocyte arginase induces cardioprotection via a mechanism that is entirely dependent on erythrocyte NOS3 (**Figure 2**).<sup>40</sup>

Interestingly, arginase is upregulated in erythrocytes in type 2 diabetes - an important co-morbidity in patients with STEMI.<sup>41, 42</sup> Accordingly, it was recently demonstrated that erythrocytes from both mice and patients with type 2 diabetes markedly impair recovery of cardiac systolic function, increase left ventricular end-diastolic pressure and increase infarct size following IR in comparison with erythrocytes from control mice or healthy humans.<sup>42</sup> The underlying mechanism behind this effect was increased arginase activity in erythrocytes which led to a decrease in NO production. It further resulted in increased reactive oxygen species (ROS) production due to uncoupling of NOS3 and increased expression of NADPH oxidase (NOX2) in erythrocytes.<sup>43</sup> The ROS species hydrogen peroxide produced by erythrocytes activates endothelial cell arginase and NOX1 which results in endothelial oxidative stress and impaired endothelium-dependent relaxation.<sup>43</sup> Thus, available data suggest that erythrocytes are prominently involved in events occurring during IR by export of NO bioactivity under strict control of erythrocyte arginase. Furthermore, erythrocytes are important targets for cardioprotective therapies including arginase and ROS inhibition. In addition, erythrocyte NOS/NO bioactivity has been suggested to be associated with RIC via increased erythrocyte deformability.<sup>44</sup> Finally, erythrocyte dysfunction characterized by increased arginase activity and ROS production leading to endothelial dysfunction aggravates IR injury and increases infarct size in type 2 diabetes.<sup>42</sup> Therefore, erythrocytes represent an important potential target for cardioprotection.



#### 4. Extracellular vesicles and circulating miRNA

In addition to circulating cells, blood contains large numbers of EVs.<sup>45-48</sup> Most of these EVs originate from platelets and erythrocytes, but they are also produced by other circulating cells such as leukocytes, and by vascular cells particularly the endothelium. Historically, the cardiovascular field has focussed on the population of larger EVs, called microvesicles (MVs), in part because they are relatively easy to study using methods such as flow cytometry. Over the past few years, there has been increasing interest in the smaller type of EVs called exosomes, particularly because of their apparent signalling role.<sup>45-48</sup> As we discuss these different types of EVs below, it is important to be aware that results can be highly dependent on the isolation and purification methods used, and that the purity and specific fractions of exosomes achieved using commonly used isolation methods can be quite variable.<sup>46, 48, 49</sup> Furthermore, while EVs certainly contain miRNA, miRNA is also found in the blood complexed to lipoproteins and Argonaute proteins, and the relative importance of these different vehicles for the transfer of miRNA is highly debated.

##### i. Exosomes

Exosomes are nano-sized (50-150 nm diameter) lipid bilayer vesicles released from cells when multivesicular bodies fuse with the plasma membrane.<sup>46, 48</sup> Exosomes are secreted by all cell types and act as universal propagators of intercellular communication. Since high concentrations of exosomes are found in the blood ( $\sim 10^{10}$  per ml<sup>50</sup>), they have been hypothesized to mediate the transmission of the cardioprotective signal of RIC.<sup>51</sup> Indeed, RIC was shown to increase the concentration of exosomes in the blood,<sup>50</sup> and in 2014, the first evidence that cardioprotection by RIC might be transmitted by EVs, most likely exosomes, was obtained.<sup>52</sup> In this study, pre-treatment with exosomes from conditioned donor hearts attenuated infarct size in non-preconditioned recipient hearts undergoing IR.<sup>52</sup> Recently it has been shown that exosomes derived from the plasma of rats subjected to RIC play a role in reducing oxidative stress-mediated injury.<sup>53</sup>

Platelets are a major source of circulating EVs, releasing both exosomes and microvesicles.<sup>48, 54</sup> Platelet EVs are also present in atherosclerotic plaques.<sup>55</sup> Several stimuli can augment the release of EVs, including physical-chemical stresses and pro-apoptotic stimuli. Platelet exosomes appear to have an anti-thrombotic effect.<sup>45</sup> Platelet-derived EVs can transfer RNAs to recipient cells and influence their activity.<sup>45, 56</sup> While studies suggest an important role for platelet-derived miRNAs in haemostasis, thrombosis, and unstable coronary syndromes,<sup>56</sup> it is less clear whether miRNA, either from platelets or EVs, could act rapidly enough to influence acute infarct formation after IR.

Exosomes are increasingly being exploited for their therapeutic cardioprotective role in progenitor/stem cell-based therapy.<sup>46</sup> Molecules and EVs secreted by progenitor cells appear to create a reparative and regenerative milieu in the tissue microenvironment, which may be more important than the differentiation potential of the cells themselves. Exosomes purified from culture medium conditioned by resident cardiac progenitor cells (Exo-CPC), but not exosomes released from normal dermal fibroblasts, are cardioprotective and proangiogenic *in vivo*.<sup>57, 58</sup> Exo-CPC injected into the infarct border zone reduced scar size, increased viable mass and vessel density, and improved global heart function after MI in mice.<sup>57, 58</sup> Not only CPC-derived exosomes can induce a cardioprotective signal, since differences are observed when comparing CPC with exosomes from patient-matched, bone-marrow derived, mesenchymal stem cell (BMC). Although Exo-BMC provide some cardioprotection after acute MI, they are not as effective as Exo-CPC.<sup>59</sup>

The exact mechanism by which exosomes protect cardiomyocytes from IR injury has yet to be elucidated but it may involve the exosome's cargo of mRNA, short non-coding RNA (miRNAs, Y-RNA) and/or proteins.<sup>57, 58, 60</sup> Given their abundance and specific expression within tissue-specific exosomes, miRNAs appear to be an important component, although there are some aspects that are



not yet clear, including how transferred miRNAs are incorporated into an endogenous RISC complex and mediate their effect in competition with large amounts of host miRNA.<sup>61</sup> The most highly enriched miRNAs in Exo-CPC include miR-146a-3p, miR-132 and miR-210.<sup>57, 58</sup> Gain and loss-of-function studies revealed antiapoptotic and proangiogenic properties of these miRNAs. Plasma exosomes induced by RIC transfer miR-24 and decrease oxidative stress-mediated apoptosis into cardiomyocytes by downregulating expression of the pro-apoptotic protein Bim.<sup>53</sup>

CPC cultured as 3-D cardiospheres (CDC) release exosomes that contain many short RNAs that are unique to CDCs compared to fibroblast-derived exosomes.<sup>60</sup> The most abundant RNA species found in CDC-exosomes is a Y RNA fragment (EV-YF1).<sup>60</sup> Its relative abundance in CDC-exosomes correlates with an indirect *in vivo* reduction of cardiomyocyte apoptosis, by increasing expression of the known cardioprotective cytokine interleukin 10 (IL-10) into macrophages within the ischaemic area.<sup>60</sup> CDCs-exosomes reduced infarct size 48 h after reperfusion when injected in rats subjected to 45 min coronary artery occlusion.<sup>62</sup> In this case, cardioprotection was mediated by miR-181b, as demonstrated by the loss of cardioprotection caused by miR-181b antagomir and by the fact that inert exosomes from fibroblasts, after enrichment with miR-181b, were able to reduce infarct size.<sup>62</sup> Cardioprotection was found to be related to the expression of the pro-inflammatory genes *NOS2* and *TNF*, protein kinase C  $\delta$  (PKC $\delta$ ), and increased macrophage polarization.<sup>62</sup>

The protein cargo of plasma-derived exosomes includes heat-shock protein 70 (HSP70), which plays a crucial role in pro-survival effects of circulating exosomes when used in *ex vivo*, *in vivo*, and *in vitro* settings of IR.<sup>50</sup> Extracellular exosome-mediated signal activates ERK1/2 in cardiomyocytes, which trigger toll-like receptor (TLR4) leading to phosphorylation of the cardioprotective protein HSP27.<sup>50</sup> When any of these proteins are selectively blocked, or HSP70 is absent from the surface of exosomes, the cardioprotective signal is not propagated.<sup>50</sup> Diabetes impairs the cardioprotective activity of exosomes.<sup>63</sup> However, exosomes from non-diabetic rats retained the ability to protect cardiomyocytes from diabetic rats, indicating that exosome therapy can still be effective despite the hyperglycaemic environment found in diabetic patients.<sup>63</sup> Among the most highly expressed protein on Exo-CPC is pregnancy-associated plasma protein-A (PAPP-A), a protease that releases active insulin growth factor 1 (IGF-1), a key cardioprotective agent. PAPP-A appears to be required for Exo-CPC to improve functional recovery after permanent coronary artery occlusion.<sup>59</sup>

Small animal studies suggest that exosomes could revolutionize medicine due to their potent effects on cell behaviour including cardioprotection. However, there is a long route to the final goal of clinical benefits in patients using exosome-based therapeutics.<sup>48</sup> Little is known about what is the most active fraction of collected samples for exosome studies. Improved techniques for the isolation of defined size-ranges of exosome populations are needed.<sup>48</sup> It will also be important to develop methods to target exosomes to the heart to limit their potential side effects on other tissues. Finally, although some research groups have recently begun to approach the technical challenge of isolating GMP (good laboratory procedures)-grade exosomes,<sup>64</sup> several technical and regulatory aspects will need to be overcome to enable the large-scale production of exosomes.<sup>48</sup> Furthermore, pre-clinical large animals studies will be necessary before exosomes can be considered as a realistic therapeutic approach for cardioprotection.

## ii. Microvesicles

Microvesicles (MVs), also known as microparticles or ectosomes, are a heterogeneous population of EVs formed by outward budding and/or shedding of the plasma membrane. This process can occur in several cell types, including endothelial cells, erythrocytes, leucocytes, platelets and cardiomyocytes.<sup>45, 48</sup> MVs are also heterogeneous in their size and molecular composition.<sup>45, 48</sup> Although initially considered plasma membrane fragments emanating from platelets as part of the coagulation process,<sup>65</sup> it is now established that MVs are important players in intercellular

communication, since they can convey proteins, lipids, nucleic acids and other molecules with biological activity such as cytokines, hormones and coagulation factors between distant cells.<sup>66</sup> Circulating MVs have been implicated in several physiological functions such as the coagulation process, reticulocyte maturation, angiogenesis, tissue repair and inflammation.<sup>46, 48, 66</sup> The concentration of MVs in the plasma is estimated to be  $\sim 2\text{--}4 \times 10^8$  per ml.<sup>66</sup> In healthy subjects, the majority are of platelet origin as indicated by the presence of CD41, while the remaining MVs derive from granulocytes, ECs, erythrocytes and monocytes.<sup>66</sup> In contrast to the beneficial effects of platelet exosomes, noted above, platelet microvesicles can promote interactions between platelets, endothelial cells, and monocytes favouring atherogenesis.<sup>45, 47, 66, 67</sup> Since microvesicles contain procoagulant platelet membrane components, they can potentiate the coagulation response.<sup>45</sup>

The number of circulating MVs increases in patients with heart failure and vascular inflammation, most likely due to platelet activation.<sup>45, 48</sup> Moreover, the number of circulating procoagulant MVs is elevated in patients with acute coronary syndrome and chronic ischemic heart disease.<sup>48</sup> Endothelial-derived MVs can increase in heart failure, hypertension, coronary artery disease and carotid artery disease, possibly due to endothelial injury and dysfunction.<sup>68</sup>

In addition to their importance as biomarkers, MVs elicit biological responses in recipient cells which may depend on the cell-type and of origin as well as its physiological status.<sup>48</sup> For example, platelet-derived MVs injected into the myocardium induced angiogenesis and stimulated post-ischaemic revascularization in a rat model of MI.<sup>69</sup> IPC increased the number of circulating MVs derived from platelets, endothelial cells and erythrocytes, and administration of these MVs significantly alleviated damage to the myocardium and restored cardiac function after IR injury by inhibiting endoplasmic reticulum stress.<sup>70</sup> MVs from mesenchymal stem cells overexpressing GATA-4 were found to be cardioprotective, and this was attributed to an increase in miR-221 levels in the MVs, which were taken up by cardiomyocytes and silenced the pro-apoptotic protein PUMA.<sup>71</sup>

On the other hand, IR may cause the release of MVs that are more damaging. MVs released from endothelial cells after IR were pro-apoptotic and pro-oxidative to cardiomyocytes.<sup>72</sup> Furthermore, MVs originating from cardiomyocytes and endothelial cells following acute MI can also be internalized by infiltrating monocytes and regulate local inflammatory responses.<sup>73</sup>

### iii. [Non-vesicular non-coding RNAs](#)

The majority (98%) of RNA molecules in the body are noncoding RNA molecules.<sup>74, 75</sup> These include ribosomal RNA, transfer RNA, microRNAs (miRNAs), long noncoding RNAs (lncRNAs) and circular RNAs (circRNAs).<sup>74</sup> miRNAs are single-stranded RNA molecules, 21-23 nucleotides in length that affect gene expression by binding to particular mRNAs and promote their degradation or inhibiting their translation into proteins. lncRNAs and circRNAs regulate the expression of genes via a complex array of epigenetic, post-transcriptional and translational modes. The effects of miRNAs are sequence-specific, but each miRNA can affect numerous mRNA molecules and each mRNA can be affected by numerous miRNAs. Since noncoding RNA molecules are involved in “fine tuning” of the expression of proteins in numerous signalling processes in the body, there is great interest in developing approaches to administer them systemically as therapeutic agents.

The miR-15 family was reported to be detrimental in IR.<sup>76</sup> In this study, a locked nucleic acid–modified (LNA)–anti-miR complementary to the seed region of the miR-15 family (LNA-miR-15), administered intravenously at the onset of reperfusion, limited infarct size in mice subjected to 75 min ischaemia followed by 24 h reperfusion.<sup>76</sup> Targeting miR-15 also prevented the decrease in Pdk4 (a key regulator of mitochondrial function) and Sgk1 (an inhibitor of cardiomyocyte apoptosis).<sup>76</sup>

In pigs subject to 60 min ischaemia followed by reperfusion, administration of LNA-miR-92a 5 min prior to reperfusion reduced infarct size and left ventricular function improved.<sup>77</sup> However, a benefit was only seen after catheter-based delivery, and not by intravenous infusion. LNA-92a also increased capillary density and decreased leukocyte infiltration and cardiomyocyte cell death.<sup>77</sup>

In rabbits, intravenous administration of liposomal-encapsulated miR-145 immediately after reperfusion (following 30 min coronary artery occlusion) reduced myocardial infarct size and improved left ventricular function two weeks after infarction.<sup>78</sup> The target of miR-145 was found to be fibroblast growth factor receptor substrate 2, and its effect was at least partially mediated by the activation of autophagy.<sup>78</sup>

Following physical or pharmacological interventions, changes in the expression of certain noncoding RNAs might be expected to reveal those that represent the most promising targets. Several studies have explored which noncoding RNAs are affected by IR or interventions such as IPC and IPost and have tested whether these noncoding RNAs have protective effects in experimental models.<sup>4</sup>

Using unbiased miRNA omics approach, several miRNAs were identified that affected by IPC and IPost and termed these miRNAs protectomiRs. Transfection of protectomiRs (specific miRNA mimics or antagomirs as appropriate) into cardiac myocytes validated their cardiocytoprotective efficacy. In particular, a miR-125b\* mimic was shown to be of high relevance for cardioprotection.<sup>79, 80</sup> As expected, the concentration of numerous noncoding RNA molecules is altered by ischaemia, IR, conditioning stimuli and medications. Several group have shown that by offsetting these changes with specific agonists or antagonist, the protective effects of various interventions are lost. For example, inhibiting miR-499 abolishes the protective effect of post-conditioning;<sup>81</sup> the protective effect of pioglitazone *in vitro* against simulated IR is dependent on downregulating miR-29 levels. Hence, 3-day pre-treatment with antagomirs against miR-29a or 29c attenuated apoptosis and limited infarct size in an *in vivo* rat model of 30 min ischaemia/24 h reperfusion.<sup>82</sup>

Another example of a cardioprotective miRNA is miR-21. In an isolated heart model, infarct size was smaller when mice had been pre-treated 24 h previously with synthetic miR-21.<sup>83</sup> However, miR-21 also appears to contribute to remodelling and fibrosis in the failing heart.<sup>84</sup>

Although this review focusses primarily on studies in which noncoding RNAs or their antagonists was administered around the time of reperfusion because of their relevance to patients with STEMI, it should be noted that benefit for RNA-based therapies has also been seen in models of permanent coronary artery ligation and/or when treatment is administered prior to the onset of ischaemia.<sup>75</sup>

In general, the use of noncoding RNA based-therapy as a short-term therapy to mitigate IR injury and reduce infarct size in patients presenting with STEMI requires rapid and specific delivery of the RNA molecules to the heart, and a rapid onset of action.<sup>4</sup> In the clinical setting such therapeutic agents should either be given intravenously during the ischaemic phase or intravenously or intra-coronary during primary percutaneous coronary intervention, and must be able to enter the cells rapidly and have a rapid effect gene expression. Approaches are being developed to aid intracellular RNA delivery or chemically modify RNA to permit its direct cellular uptake.<sup>85, 86</sup> However, clinical translation of these pharmaceutical agents is still at an early stage.

## 5. [Thrombosis and blood clotting factors](#)

Several interventions targeting blood clotting factors have been found to limit infarct size independently of the haemostatic function of these proteins. Blood coagulation is initiated when plasma factor VII (FVII) binds to its cellular receptor tissue factor (TF), expressed on deeper cell layers of the vessel wall, and is converted to the active protease FVIIa (extrinsic initiation

pathway)(**Figure 3**).<sup>87</sup> The TF:FVIIa complex activates the factors IX and X resulting in the generation of only minute amounts of thrombin that immediately start to amplify its own production by activating factors XI, VIII and V. These reactions of the intrinsic pathway result in the mass production of thrombin, now available to induce fibrin formation and blood clotting. Additionally, thrombin activates platelets, endothelial cells, and cardiomyocytes, by cleavage of protease activated receptors (PARs) and the subsequent activation of intracellular signalling pathways.<sup>88</sup> The intrinsic regulation of blood coagulation is achieved by the thrombin-induced generation of the thrombomodulin-protein C pathway, resulting in an effective control of thrombin generation.

TF initiates the clotting cascade and is a major prothrombotic factor.<sup>89</sup> Increased plasma TF levels, associated with EVs, are observed in patients with AMI, reflecting enhanced intravascular procoagulant activity.<sup>89</sup> Experimental studies indicate that cardiac IR increases TF activity. *In vivo* studies in rabbit models have shown that anti-TF therapy prevented the transient decrease in regional myocardial blood flow, reduced platelet and fibrin(ogen) accumulation, and reduced infarct size.<sup>90</sup> Thrombin may also contribute to the pathology of IR injury, since in a rabbit model of IR, selective inhibition of thrombin by recombinant hirudin (lepirudin) decreased infarct size.<sup>90</sup> The TF-thrombin pathway may also contribute to myocardial injury by an additional mechanism that is not dependent on fibrin deposition but involves activation of protease activated receptors (PARs) on vascular endothelial cells and cardiac myocytes.<sup>90</sup> Since myocardial IR injury is partly mediated by thrombin and several cellular responses to thrombin are mediated by PARs, PARs have been extensively investigated as potential targets for cardioprotection.

The four known PARs are G-protein coupled receptors that are activated by several serine proteases, including coagulation and mast cell-derived proteases. For example, thrombin cleaves and activates PAR-1, -3 and -4 on a variety of cells and thereby activates each of these receptors, whose new amino-terminal portion serves as leached ligand.<sup>91</sup> PAR-1 is the high-affinity receptor for thrombin and is expressed by several cell types in the heart, including cardiomyocytes and cardiac fibroblasts. Since PAR-1 is expressed as a “cell-bound substrate” of thrombin on both platelets and immune cells, hormonal doses of the enzyme are sufficient to provoke platelet aggregation or a variety of immune responses in the context of inflammation and cardiovascular disease.<sup>92</sup> Treatment of rats or isolated hearts with a selective PAR-1 antagonist, reduced infarct size in a dose-dependent manner and increased ventricular recovery following IR.<sup>93</sup> When PAR-1 is cleaved by thrombin it releases a 41-amino-acid peptide called parstatin. Both parstatin and its putative signal peptide (N-terminal fragment 1-26), reduced infarct size when administered to rats prior to IR<sup>94, 95</sup>. The underlying mechanisms may involve the known cardioprotective pathways including the RISK and the MPTP pathway.<sup>95</sup>

Activated protein C (APC) is a serine protease that serves as natural anticoagulant with an important role in regulating thrombin formation and the extent of fibrin formation. It is recognized by the endothelial protein C receptor and alters signalling of the thrombin-PAR-1 complex. In a mouse model of acute IRI, administration of APC significantly reduced myocardial infarct size,<sup>96-98</sup> with PAR-1 required for this process.<sup>97</sup> Interestingly, a variant APC, lacking catalytic activity, remained protective, implying that the protection from IRI is independent of its proteolytic activity.<sup>96</sup> Furthermore, infarct size reduction depended on its PAR-1 signalling, but not its anticoagulant properties.<sup>98</sup>

Although APC serves as a major ligand/activator of PAR-2 on immune cells, its role in IRI is controversially discussed and the protease(s) that activate PAR-2 during cardiac IRI are not known.<sup>88</sup> Infarct size was significantly reduced in PAR-2<sup>-/-</sup> mice subjected to 30 min ischaemia and 2 h reperfusion, as well as exhibiting decreasing oxidative/nitrative stress.<sup>99</sup> In contrast, infusion of a PAR-2-activating peptide reduced infarct size in isolated perfused rat hearts.<sup>100</sup> Interestingly, this peptide showed additive protection with an IPC protocol of 2 min ischaemia followed by 10 min

reperfusion.<sup>101</sup> Furthermore, the PAR-2 agonist peptide SLIGRL reduced infarct size when administered to rats at the time of reperfusion, via a pathway involving ERK1/2 and PKC.<sup>102</sup> SLIGRL also reduced infarct size in isolated rats hearts, via pathways involving PKC $\epsilon$  or PKA, and transient receptor potential vanilloid type 1 -dependent release of calcitonin gene-related peptide and substance P.<sup>103</sup>

PAR-4 knockout mice exhibited reduced infarct size after acute IR. This may be due to protection from a Src- and epidermal growth factor receptor-dependent pathway of JNK-induced apoptosis.<sup>104</sup> Two structurally unrelated PAR-4 antagonists reduced infarct size in rats when administered prior to ischaemia either *in vitro* or *in vivo*, via a mechanism that appears to involve adenosine.<sup>105</sup>

Plasmin is the main enzyme that dissolves fibrin blood clots. The main function of plasminogen activator inhibitor type-1 (PAI-1 or SERPIN E1) is to oppose the plasmin activation cascade, thereby maintaining the clot. Increased expression of PAI-1 is profibrotic in hearts subjected to MI. A markedly greater extent of infarction was observed in PAI-1 knockout mice compared with controls and this was associated with haemorrhage and inflammation.<sup>106</sup>

Overall, pharmaceutical agents targeting thrombosis and blood clotting factors appear have multiple benefits in the setting of IR, including benefits independent of haemostasis, although these are not always easy to completely separate mechanistically.

## 6. [Conclusion](#)

As can be seen from this review, circulating cells and factors can strongly impact IR injury via various mechanisms. Erythrocytes, for example, can export NO bioactivity and be cardioprotective. Platelets, in addition to their role in haemostasis and thrombosis, secrete a large number of factors that can influence the development of IR injury both positively and negatively. Erythrocytes, platelets and other cell types can release both MVs and exosomes which may have both detrimental or protective characteristics in the setting of IR. These effects may be mediated by the transfer of miRNA to cardiomyocytes, or through ligand-receptor signalling or other mediators (e.g.: NO).

In many of the experiments described, the end target is likely to be the cardiomyocyte, since ultimately, it is these cells that must be preserved in order to limit infarct size and retain contractile function. However, there are other important aspects to IR injury such as endothelial damage and microvascular obstruction which may be targets. Furthermore, the interactions between thrombus, clotting factors and circulating hematopoietic cells have not yet been clarified in terms of IR injury. With greater understanding of PAR-1 and PAR-4 signalling pathways and their role in IR injury may come opportunities for better tailored therapies to prevent tissue injury.

One firm conclusion that can be drawn is that it is important to consider the interaction of potential cardioprotective agents with co-medications such as platelet inhibitors, since these appear to have cardioprotective actions independent of their role in haemostasis. Furthermore, co-morbidities such as diabetes can impact not only the induction of cardioprotection in the target cardiomyocytes, but can also influence the function of platelets, erythrocytes and EVs, and consequently impair their ability to mediate cardioprotection. Finally, future studies of potential cardioprotective agents should consider not just their direct effect on cardiomyocytes, but on indirect effects that may be mediated via circulating blood cells and factors.

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## 9. Conflict of Interest

PF is the founder and CEO of Pharmahungary Group, a group of R&D companies. IA, LB, YB, HACF, MVC, SD, JMD, HG, CP, JP, PP and KTP declare no conflicts of interest.



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## 11. [Figure legends](#)

**Figure 1.** Ischaemia and reperfusion causes the activation of platelets, which subsequently release a multitude of factors with divergent effects on infarct size. These include exosomes and microvesicles (both types of extracellular vesicles), SDF-1a, chemerin, sphingosine-1 phosphate, and platelet activating factor (PAF). P2Y<sub>12</sub> inhibitors can prevent platelet activation and can also reduce infarct size. See text for details.

**Figure 2.** Erythrocytes contain endothelial nitric oxide synthase (NOS3) which protects the heart via the production of nitric oxide (NO), S-nitrosothiols (S-NO) or nitrite. Since NOS3 competes with arginase for the common substrate arginine, inhibition of arginase can be cardioprotective.

**Figure 3.** Initiation, amplification and feedback anticoagulant mechanisms in the coagulation cascade. The different phases, from initiation of coagulation due to exposure of tissue factor and binding of its ligand factor VII/VIIa either at a wound/extravascular site or in the intravascular compartment (microvesicles), designated as “extrinsic pathway”, to amplification and production of thrombin by the positive feedback reactions of the “intrinsic pathway” are indicated. In parallel to fibrin clot formation, the majority of thrombin will distantly bind to its endothelial cell receptor thrombomodulin to induce the activation of protein C (PC) into activated protein C (APC), which limits further thrombin production by degrading the procoagulant cofactors VIIIa and Va. While these reactions are sufficient to achieve wound healing upon physiological haemostasis, when an atherosclerotic plaque ruptures, thrombogenic substrates are exposed that can initiate (auto-) activation of the factor XII-dependent reactions of the contact phase, resulting in enhanced thrombin generation and hence, fibrin clot formation and eventually thrombosis. The inhibitors mentioned in the text are indicated in red.







